Sustained Human Hematop iesis in Sheep Transplanted In Utero During Early Gestati n With Fracti nated Adult Human Bone Marrow Cells

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Sheep were transplanted in utero during early gestation with subpopulations of adult human bone marrow (BM) cells enriched for human progenitor and hematopoietic stem cells (HSC). Chimerism was documented in three of seven transplanted fetuses using monoclonal antibodies against human-specific hematopoietic cell lineages and/or cytogenetic analysis of BM and peripheral blood cells of recipients. Only chimeric sheep BM cells expressing CD45 (6.0% of total BM cells) formed human hematopoietic colonies in response to human recombinant cytokines as determined by cytogenetic analysis. Sorted CD45⁺ BM cells developed human T-cell colonies containing CD3⁺, CD4⁺, and CD8⁺ cells. DNA from chimeric BM cells obtained 3 months after birth displayed a finger printing pattern identical to that of DNA from the human donor of the HSC graft. These studies indicate that first trimester sheep fetuses are tolerant of adult human HSC grafts, thus permitting the creation of xenogeneic chimera expressing human myeloid and lymphoid lineages. The present findings also suggest that HSC grafts from immunologically competent, HLA-mismatched adult donors may be useful for correcting human genetic diseases in utero during early gestation.

THE FETUS REPRESENTS the ideal host for hematopoietic stem cell (HSC) transplantation because of its ontologic readiness and its tolerance of foreign grafts. Alternatively, to achieve successful fetal engraftment with HSC, the graft should be devoid of immunologic elements capable of provoking graft-versus-host disease (GVHD). Fetal liver cells, which at an early gestational age are immunologically "naive," have been successfully used to establish hematopoietic chimeras after in utero allogeneic transplantation of preimmune recipients.[1,2] In this report, we evaluated the suitability of subpopulations of adult human marrow as grafts for in utero transplantation. Fetal sheep (gestation period, 145 days), like many mammals, including humans,[3] have been shown to be preimmune during the first trimester[4] and therefore offer a 50-day window during which the usual immunologic barriers to marrow transplantation might not be operable. Human in utero HSC transplants could be theoretically used to reverse or correct a number of congenital diseases. Such an intervention could be successful, especially if it is performed while the fetus is still preimmune and if the bone marrow (BM) graft lacks immunologically competent cells that may cause GVHD.

Our laboratory[5-7] and that of others[8] has been examining the self-renewal and differentiative capacities of a small fraction of adult human BM consisting of cells expressing CD34 and undetectable or low levels of the class II major histocompatibility complex antigen HLA-DR (CD34⁺ HLA-DR⁻ cells). CD34⁺ HLA-DR⁻ cells have been f und to contain the colony-forming unit-blast (CFU-B1), which is a primitive cell capable of self-renewal and differentiation into multiple hematopoietic lineages,[6] the burst-forming unit-megakaryocyte (BFU-MK), which is a primitive megakaryocyte progenitor cell,[9] as well as the high proliferative potential–colony-forming cell (HPP-CFC), a primitive myeloid progenitor cell.[7] In addition, CD34⁺ HLA-DR⁻ cells have been shown to be capable of initiating and sustaining long-term in vitro human hematopoiesis,[5,7,8] indicating that the human HSC or a cell that possesses many functional characteristics of the human HSC is likely present among the CD34⁺ HLA-DR⁻ BM cells.

It was reasoned that if subpopulations of human BM cells that actually contained primitive pluripotential HSC w re used as marrow grafts, immunologic tolerance to sheep self-antigens might be induced in progeny cells if th se human cells were given a chance to differentiate and mature in a "non-self" permissive environment. We report here that transplantation of fractionated adult human BM cells enriched for hematopoietic progenitor and stem cells into seven immunologically immature sheep fetuses resulted in the creation of three hematopoietic xenog nic chimeras, as evidenced by the analysis of BM cells from two fetuses killed in utero and from one animal after birth. Chimerism was sustained 3 months after birth (6 months after engraftment) without any signs of GVHD.

MATERIALS AND METHODS

Cell separation. Human BM was obtained from an adult healthy male donor after obtaining informed consent according to guidelines established by the Human Investigation Committee of the Indiana University School of Medicine. Low-density BM cells separated over Ficoll/Hypaque were suspended in phosphate-buffered saline (PBS)-EDTA (PBS, pH 7.4, containing 5% fetal calf serum, 0.01% EDTA wt/vol, and 1.0 g/L D-glucose) and loaded into a standard chamber of an elutriator system (Beckman Instruments, Inc, Palo Alto, CA) previously sterilized with 70% ethanol.[6] Effluent samples eluting at 11, 12, 14, 16, 18, 20, and 22 mL/min were collected. Fractions eluting at 12 and 14 mL/min (Fr 12-14) were pooled and used for subsequent steps.

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Immunofluorescence staining and cell sorting. Fr 12-14 BM cells were first stained with CD34 (HPCA-1, IgG_1 isotype) over ice for 20 minutes. Phycoerythrin (PE)-conjugated HLA-DR and isotype-specific, fluorescein isothiocyanate (FITC)-conjugated goat-antimouse IgG_1 second step antibody (Southern Biotechnology, Birmingham, AL) were then added for another 20 minutes. The cells were then washed and sorted on a Coulter Epics 753 flow cytometer (Coulter Electronics, Hialeah, FL) as previously described.[10] Positivity for each fluorochrome was defined as fluorescence greater than 99% of that of the controls. This staining protocol allows for the separation of $CD34^+$ $HLA\text{-}DR^-$, $CD34^+$ $HLA\text{-}DR^+$, $CD34^-$ $HLA\text{-}DR^+$, and $CD34^-$ $HLA\text{-}DR^-$ cells from within the Fr 12-14 BM cells. In these experiments, $CD34^+$ $HLA\text{-}DR^-$ cells were collected separately ($CD34^+$ $HLA\text{-}DR^-$ cells) while the remaining three phenotypes were collected together to constitute what will be referred to in this report as complementary cells. Cell viability after sorting was greater than 98%. Separation of $CD45^+$ and $CD45^-$ chimeric BM cells was achieved by labeling low density chimeric BM cells obtained by separation over Ficoll/Hypaque with FITC-conjugated CD45 and then isolating $CD45^+$ and $CD45^-$ cells by cell sorting as described above. Phenotypic analysis of chimeric and control low-density sheep BM and PB cells and cultured T cells was performed on a FACScan (Becton Dickinson Immunocytometry Systems, San Jose, CA) using directly conjugated monoclonal antibodies (MoAbs). All MoAbs were obtained from Becton Dickinson Immunocytometry Systems.

In utero transplantation. BM graft cells were injected into seven 42- to 48-day-old sheep fetuses intraperitoneally through a 22-gauge needle as previously described.[1] Briefly, the uterus of pregnant ewes was accessed through a midline laparotomy incision. A transverse incision was then made through the myometrium and chorion. The BM graft was injected intraperitoneally into the fetus after manipulating it into an amniotic bubble. The myometrium was then closed in a double layer and the pregnancy allowed to proceed to term except in two fetuses that were killed in utero 30 days later.

Hematopoietic progenitor cell assays. Cells assayed for the generation of hematopoietic colonies were obtained from the BM of a control sheep, the human donor of the graft, and the chimeric sheep (sheep 4038) as well as $CD45^+$ and $CD45^-$ BM cells from sheep 4038 isolated by flow cytometric sorting. Also, a mixture of 95% control sheep and 5% normal human BM cells was prepared and assayed for hematopoietic progenitor cells. Progenitor cell assays were performed as previously described.[7] Briefly, cells at various concentrations were suspended in 35-mm plastic tissue culture dishes containing 1 mL of 30% fetal bovine serum (FBS), 5×10^{-5} mol/L 2-mercaptoethanol, 1.1% methylcellulose in Iscove's Modified Dulbecco's Medium, and either 1 U human purified erythropoietin (Epo; 50 U/mg protein; Toyobo Co Ltd, Osaka, Japan) or 1 ng interleukin-3 (IL-3) plus 1 ng granulocyte-macrophage colony-stimulating factor (GM-CSF). IL-3 and GM-CSF were obtained from Genzyme Corp (Boston, MA). The cultures were incubated at 37°C in a 100% humidified atmosphere of 5% CO_2 in air. Erythropoietic colonies (CFU-E) were enumerated after 6 days, whereas erythropoietic bursts (BFU-E) were counted after 9 and 14 days of incubation. Granulocyte-macrophage colonies (CFU-GM) were enumerated after 9 and 14 days using standard criteria for their identification.[6] The reported data reflect the number of colonies per 5×10^4 plated cells and represent the mean ± SE of assays performed in duplicate.

Karyotypic analysis. To analyze peripheral blood (PB), BM, or cultured cells cytogenetically, the available number of cells was treated in two different ways. Fresh cells were first cultured in 10 mL RPMI 1640 + 20% FBS, stimulated with 0.12 μg phytohemagglutinin (PHA-HA15; Wellcome Diagnostics, Research Triangle Park, NC), and maintained for 72 hours in 5% CO_2 at 37°C, then treated with colcemid as described below. Cells obtained from hematopoietic colonies or from cultured T cells were directly treated after harvesting with colcemid. Colcemid was added at 0.18 μg/mL for 35 minutes. The cells were then treated with 0.075 mol/L KCl hypotonic solution for 15 minutes and fixed in 3:1 methanol:acetic acid. Cells were dropped onto cold wet slides and GTG banded.

Propagation of T lymphocytes in vitro. Propagation of T lymphocytes from sorted chimeric $CD45^+$ and $CD45^-$ BM cells was performed as previously described.[11] Briefly, round-bottomed 96-well plates were seeded with 10^4 irradiated (5,000 rad) cells of the Epstein Barr virus (EBV)-transformed B-cell line JY suspended in RPMI 1640 containing 10% FBS, 5×10^{-5} mol/L 2-mercaptoethanol, 1% final concentration PHA-HA15 (Wellcome Diagnostics), and 5% vol/vol final concentration of T-cell growth factor (TCGF; Cellular Products, Buffalo, NY). Responder cells ($CD45^+$ or $CD45^-$ chimeric BM cells) were added at 50 cells per well. The plates were incubated at 37°C in a 100% humidified atmosphere of 5% CO_2 in air for 14 days and were fed with 10 μL/well of TCGF on day 7. Wells seeded with either $CD45^+$ or $CD45^-$ BM cells that contained colonies were harvested, pooled, and the cells stained for flow cytometric immunofluorescence analysis as described above.

Variable number tandem repeat polymorphism analysis. DNA was isolated from BM cells from the chimeric sheep at 3 months after birth, BM cells of a control sheep, and the donor of the human BM graft according to published methods.[12] DNA was digested by *Hae* III and electrophoresed on a 1% agarose gel. The gel was then dried and probed directly[13] with radiolabeled pYNH24 (courtesy of Dr Y. Nakamura, University of Utah) and MS31 (Cellmark Diagnostics, Germantown, MD) using a random primed DNA labeling kit (Boehringer Mannheim, Indianapolis, IN).

RESULTS

A total of seven sheep fetuses with gestational ages of between 42 and 48 days were transplanted in utero with fractions of adult human BM cells.[1] Table 1 summarizes th outcome of these transplants. Two animals (4035 and 4036) received 2×10^6 $CD34^+$ $HLA\text{-}DR^-$ cells and 10^6 irradiated (5,200 rad) complementary cells (see Materials and Methods). The complementary cells were injected with the $CD34^+$ $HLA\text{-}DR^-$ cells to act as "carrier" cells that would facilitate the homing of unaltered $CD34^+$ $HLA\text{-}DR^-$ cells to the liver or BM. The remaining five animals received unaltered cells from one fraction of cells only or a combination of cells from both fractions. The first test of whether engraftment had been accomplished was conducted approximately 30 days posttransplantation in two animals killed in utero. By karyotypic analysis of more than 200 BM cells, it was determined that 3.8% of total BM cells in sheep 4035 at 1 month after receiving the graft were of human origin (Table 2). Similarly, fetus 4033, which was transplanted with 2×10^6 $CD34^+$ $HLA\text{-}DR^-$ cells without complementary cells, was found to be a chimera 1 month posttransplantation with 1.5% of the BM cells being of human origin. It was thus calculat d that a single femur from sheep 4035 contained 6.5×10^5 human cells, while that of sheep 4033 contained 3.6×10^5 human cells. These numbers of human cells detect d in a single femur from each fetus represent 32- and 18-fold increases over the number of viable human

Table 1. Summary of the Outcome of Transplanting Seven Sheep Fetuses With Fractionated Adult Human BM Cells

Sheep Fetus Identification No.	Gestational Age When Transplanted (d)	No. and Phenotype of Graft Cells		Outcome	Engraftment
		$CD34^+$ $HLA\text{-}DR^-$	Complimentary Cells		
4033	42	2×10^4	—	Killed† day 72	+
4034	44	2×10^4	—	Born alive	−
4035	45	2×10^4	$1 \times 10^{6*}$	Killed† day 72	+
4036	48	2×10^4	$1 \times 10^{6*}$	Born alive	−
4037	46	4×10^4		Died in utero	
4038	46	2×10^4	1×10^6	Born alive‡	+
4039	43	—	10×10^6	Died in utero	

*Cells were irradiated (6,200 rad) before injection.

†These two animals were killed 1 month after receiving the human BM graft. Kryotypic analysis of BM cells extracted from the femur showed the presence of 1.5% and 3.8% human cells in the BM of fetuses 4033 and 4035, respectively.

‡Sheep 4038 is the animal studied in detail in the rest of the report.

BM cells originally transplanted into fetuses 4035 and 4033, respectively. We have no way of estimating the total number of human cells in the whole animal. Our estimates of 32- and 18-fold expansion of human cells are at best a gross underestimate because we have quantitated the number of cells in a single femur only. BM cells from these fetuses were also assayed for their potential to give rise to human hematopoietic colonies (see below for details). When cytogenetic analysis of cells from 14-day-old CFU-GM–derived colonies was performed, only human karyotypes were detected, indicating the presence of human myeloid progenitor cells in the BM of the two fetuses.

While two additional animals died in utero, a total of three lambs were born and were therefore available for further examination. Karyotypic analysis of PB and BM samples from two of the three lambs born at term (4034 and 4036 in Table 1) did not show any evidence of engraftment. The third lamb (4038), born alive at term, was found to have engrafted with human BM cells. A detailed analysis of PB and BM samples from sheep 4038 was then conducted at 2 and 3 months postparturition (5 and 6 months posttransplantation).

Fetus 4038 was transplanted at 46 days gestational age with a combination of 2×10^4 $CD34^+$ $HLA\text{-}DR^-$ and 1×10^6 complementary (see Table 1) human adult BM cells. At 2 months after birth (5 months after engraftment), PB and BM samples from sheep 4038 were obtained for analysis. As can be seen in Fig 1, low-density BM cells from a control sheep were not reactive with any of the mouse antihuman cell surface marker MoAbs used. However, when BM cells from sheep 4038 were analyzed, it became evident that a fraction of its cells representing 6% of the low-density BM cells analyzed was reactive with the leukocyte common antigen CD45 that identifies a family of glycoproteins (180 to 220 Kd) that are expressed on the surface of a variety of human hematopoietic cells.[14] To further examine the nature of these human cells, lineage-specific MoAbs were used. Human T lymphocytes expressing either CD8 (1.1%) or CD4 (0.9%) were detected in the chimeric BM. Similarly, B lymphocytes (2% $CD19^+$ cells) and cells reactive with CD16 and CD56, and therefore presumably human NK cells,[15] were also identified. Although it was possible to detect the presence of cells expressing HLA-DR, we did not convincingly detect $CD34^+$ cells flow cytometrically. It is quite conceivable, however, that because $CD34^+$ cells normally represent on the average 1% or less of total human BM cells,[16] such a small percentage of cells (0.06% when considering that only 6% of the chimeric BM cells are human) would be difficult to detect.

Table 2. Karyotypic Analysis of Cells Derived From Hematopoietic Colonies Grown In Vitro or From Chimeric BM and PB

Source of Cells	Days Post-transplantation	Cells With Human Karyotype/Total Cells Analyzed	% Human Cells
BM: sheep 4033	30	20/531	3.8
BM: sheep 4035	27	6/412	1.5
BM: sheep 4038	150	6/94	6.4
PB: sheep 4038	150	12/87	13.8
Day 14 CFU-GM: unfractionated chimeric BM	150	3/3	100
Day 14 CFU-GM: $CD45^+$ cells from chimeric BM	150	6/6	100
T-cell colonies: $CD45^+$ cells from chimeric BM	150	4/4	100

Cells expressing human cell surface markers were also apparent among the PB cells of sheep 4038 (Fig 2). The various cell lineages, however, were present in the PB at lower percentages than what was observed in BM. Of particular interest is the fact that PB $CD4^+$ or $CD8^+$ T lymphocytes were present in numbers threefold to fivefold less than those found in BM, even though a total of 2.5% of PB cells expressed CD3. Chimeric PB also contained human natural killer (NK) cells, as evidenced by the detection of cells expressing CD16 and CD56, as well as a small percentage (0.2%) of CD19 B lymphocytes. PB cells from a control sheep were completely unreactive with th MoAbs used in these assays.

Although the use of this battery of mouse MoAbs recognizing human cell surface antigens proved to be species specific, two alternativ m thods to document the presence of human cells in the PB and BM of sheep 4038 were used to confirm th sustained engraftment. First, cytogenetic analysis was implemented to examine the karyotypes of cells present in the PB of sheep 4038 at 5 months after engraftment. Figure 3 shows two cells spotted in the same field, one being of sheep origin and the other displaying a full complement of human chromosomes. Among 87 total cells examined from this preparation, 12

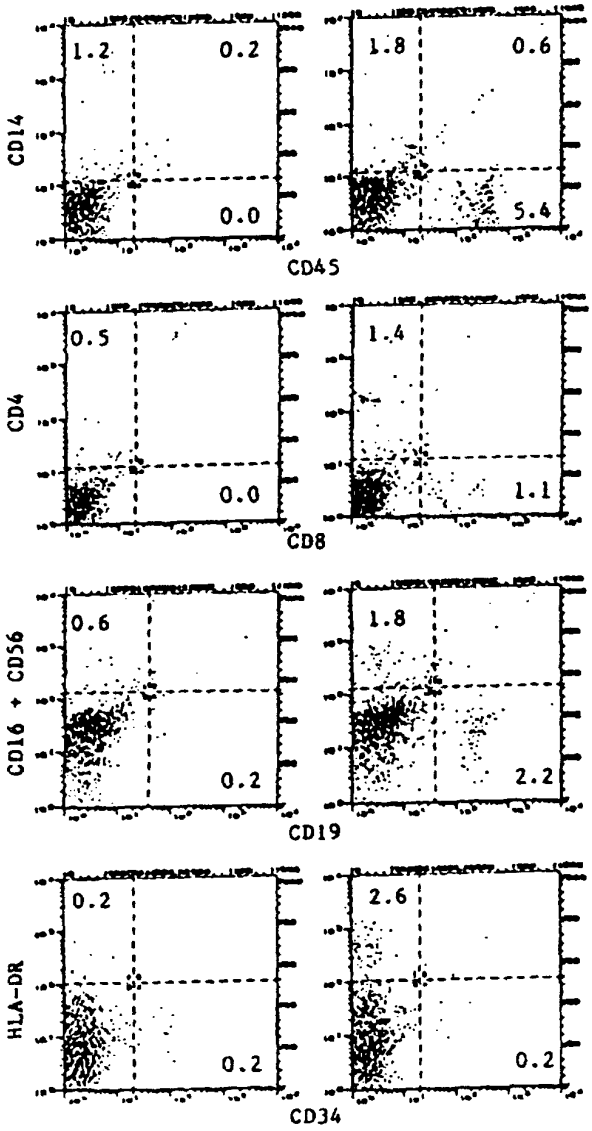

Fig 1. Phenotypic analysis of BM cells from sheep 4038 at 2 months after birth (5 months after engraftment). Low-density BM cells were separated by density centrifugation over Ficoll/Hypaque. Cells were stained over ice with FITC- and PE-conjugated MoAbs obtained from Becton Dickinson. Each dual parameter histogram generated from 10^4 events displays the distribution of FITC fluorescence on the X-axis and that of PE fluorescence on the Y-axis. The cluster designation of the MoAbs used for each set of histograms is indicated. The analysis of BM cells from a control sheep is shown in the column of four histograms on the left and that of BM cells from sheep 4038 is shown in the four histograms on the right. The percent positive events for PE, FITC, or for both simultaneously is given by the numbers appearing in the upper left (quadrant 1), lower right (quadrant 4), and upper right (quadrant 2) corners of each histogram, respectively. Quadrant 3 (lower left) contains cells negative for both FITC and PE simultaneously.

cells had a human karyotype (Table 2). In the second assay, we compared the pattern obtained in a variable number tandem repeat analysis of donor-derived BM DNA with that obtained from DNA isolated from BM cells from sheep 4038 6 months after engraftment. The results of this analysis are shown in Fig 4. As can be seen, the variable number tandem repeat pattern of DNA isolated from th BM of sheep 4038 was completely homologous to that of donor derived DNA. These data served as unequivocal proof of the persistence of chimerism in this sheep at 6 months posttransplantation.

The first assay of whether proliferation of human hematopoietic progenitor cells in chimeric BM had occurred was aimed at detecting human hemoglobin in sheep 4038. Using the different electrophoretic mobilities of human and sheep hemoglobins in a variety of gel matrices, we were unable to detect the presence of human hemoglobin in the peripheral

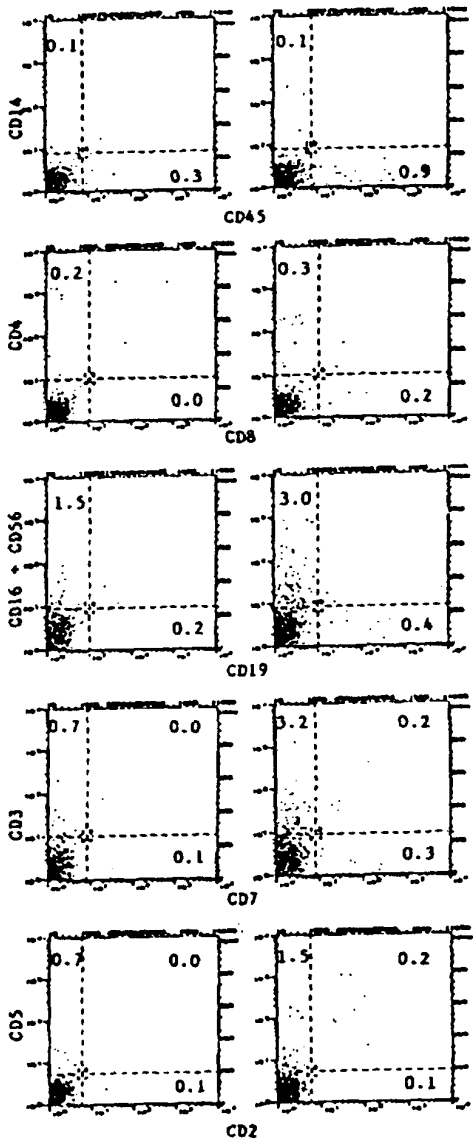

Fig 2. Phenotypic analysis of PB cells from sheep 4038 at 5 months after engraftment. The methods for cell preparation, immunofluorescence staining, and analysis were as described in Fig 1. The analysis of PB cells from a control sheep is shown in the column of five histograms on the left and that of PB cells from sheep 4038 is shown in the four histograms on the right.

Fig 3. Karyotypic analysis of PB cells from sheep 4038 at 5 months after engraftment. The figure depicts the karyotype of two cells observed in the same field. The cell in the upper left hand corner displays a full complement of human chromosomes with an arrowhead pointing to the Y chromosome (the human BM graft was from a male donor). The cell in the lower right hand corner shows a sheep karyotype.

blood of the chimeric sheep. We next proceeded to examine whether the human cells detected in the BM of sheep 4038 contained human hematopoietic progenitor cells capable of generating hematopoietic colonies in vitro. For these studies, we took advantage of the kinetics of appearance in vitro of sheep and human hematopoietic colonies. The colony formation data generated from plating chimeric BM cells from sheep 4038 at 5 months after engraftment are shown in Table 3. In the presence of recombinant human Epo, CFU-E- and BUF-E-derived colonies present in the BM of a control sheep appeared on days 6 and 9 of culture, respectively. These colonies, however, degenerated by day 14. Similarly, when cultured in the presence of human recombinant IL-3 and GM-CSF, few sheep CFU-GM-derived colonies appeared by day 9. When chimeric BM cells were cultured under the same conditions, about 10-fold and sixfold more BFU-E- and CFU-GM-derived colonies, respectively, were present on day 14 as compared with the number of colonies present on day 14 in cultures of control sheep BM cells. The presence of BFU-E-derived colonies with human BFU-E growth kinetic characteristics suggests that human erythropoiesis was established in this animal, albeit not to a sufficient level to allow detection of human hemoglobin in the PB of this animal. On day 14, cells from CFU-GM-derived colonies in cultures initiated with chimeric BM cells wer plucked, washed, and prepared for karyotypic analysis. Although the karyotype of only a few cells could be positively identified, all of these cells were of human origin (Table 2).

We wished to further confirm that, indeed, human progenitor cells were present in the chimeric BM of sheep 4038. T reach this objective, flow cytometric cell sorting was used. From 2.5 × 10^8 chimeric BM cells stained with CD45, two fractions of cells were collected: a fraction enriched for cells expressing CD45 (4.8 × 10^4 cells recovered) and a second fraction consisting of $CD45^-$ (> 3 × 10^5 cells recovered) BM cells. Both sorted fractions of $CD45^+$ and $CD45^-$ BM cells were cultured in semi-solid medium in the presence of human recombinant IL-3 and GM-CSF.[7] $CD45^+$ cells gave rise to 40 granulocyte-macrophage colonies after 14 days in culture (Table 3), whereas $CD45^-$ cells gave rise to only three such colonies. Once again, karyotypic analysis of cells plucked from CFU-GM colonies assayed from $CD45^+$ cells only showed cells with human chromosomes (Table 2).

Another yardstick by which the success of BM transplantation is measured is the capacity of the graft to establish and maintain a functional lymphoid system within the host. Flow cytometric analysis of BM and PB cells from sheep 4038 at 5 months posttransplantation indicated the presence of cells expressing CD19. To investigate whether these cells were functional or not, serum from sheep 4038 at 2 months after birth was tested for the presence of human Igs. No human Ig isotype was detected. To examine whether the human BM graft produced functional T lymphocytes in the chimeric animal, BM cells from sheep 4038 at 2 months after birth were tested for their mitogenic response in vitro. Sorted $CD45^+$ and $CD45^-$ chimeric BM cells were cultured for 14 days in the presence of IL-2 and phytohemagglutinin (PHA) over a feeder layer of irradiated EBV-transformed B cells.[11] Cells from the developing T-lymphocyte colonies were collected 14 days later and their phenotype was determin d flow cytometrically. Figure 5 depicts the results f the analysis of cells collected from T-lymphocyt colonies generated from $CD45^+$ and $CD45^-$ fractions of chimeric BM. Colonies that dev loped from $CD45^-$ T cells contained cells that failed to react with any of the human T-cell-specific MoAbs as well as with CD45 and

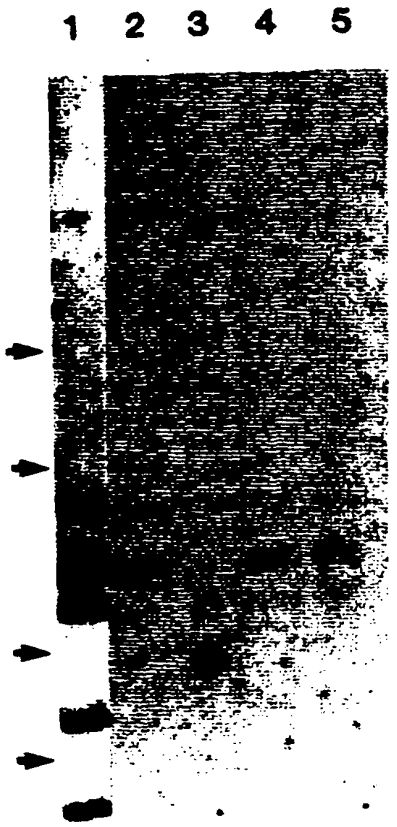

Fig 4. Variable number tandem repeat analysis of DNA isolated from the BM of sheep 4038 at 3 months after birth (6 months posttransplantation), a control sheep, and the donor of the human BM graft. The arrows on the left indicate from top to bottom 4-, 3-, 2-, and 1.6-kb markers, respectively. Lanes 1 and 2 received 10 and 0.5 μg of human donor DNA, respectively. Lane 3 received 20 μg of control sheep DNA. In lane 4, 20 μg of chimeric BM DNA was loaded. A mixture of 95% control sheep DNA and 5% donor DNA was loaded on lane 5. In making this figure, the photographic film was exposed for a longer time when photographing lanes 2 thru 5 than it was when lane 1 was photographed. A composite photograph was then constructed from the two exposures.

HLA-DR. On the other hand, $CD45^+$ BM cells gave rise to colonies containing cells greater than 98% of which were $CD45^+$ and $CD3^+$. In addition, 89.4% of these cells were positive for the expression of CD7, while 57.0% were $CD8^+$ and 30.8% $CD4^+$. As expected, most of the cells being activated with PHA and IL-2 expressed HLA-DR. Karyotypic analysis of T lymphocytes collected from colonies generated by $CD45^+$ chimeric BM cells once again showed a full complement of human chromosomes while no sheep karyotypes were detected (Table 2).

DISCUSSION

The rationale behind the experim nts reported here was provided by several previ us observations.[17-19] In 1945, Owen[17] described that vascular anastomoses between the placentas of bovine dizygotic twins allowed for the long-term retention of red blood cells of dizygotic origin in both twins. This observation led Hašek[20] to conduct his xperiments of parabiosis in chicken embryos and Billingham et al[21] to tolerize CBA mice in utero with A strain cells and then proceed to show that this acquired tolerance in CBA mice inhibited them from immunologically rejecting a skin graft from A mice. These classical experiments and several that followed[22,23] provided evidence that, early in gestation, the developing fetus is particularly tolerant to foreign antigens.

In humans, fetal thymic cells are capable of responding to mitogens by 12 weeks of gestation,[3] whereas Ig synthesis in the spleen usually does not occur until 8 weeks later.[24] Before the 12th week of gestation, the human fetus is believed to be immunologically incompetent. A similar situation exists in fetal sheep, which have been document d to be preimmune until 67 days of gestation.[22] The immunoincompetence of the mammalian fetus during early gestation makes the fetus potentially capable of being a perfect recipient of BM grafts of HLA-nonidentical donors. We took advantage of the possible readiness of sheep fetuses to accept xenogeneic HSC grafts and transplanted them in utero with subpopulations of human marrow cells to det r-mine if these fractions contain human stem cells. Several groups,[8] including ours,[5-7] have immunologically identified a phenotype of human BM cells expressing CD34 but not HLA-DR ($CD34^+$ $HLA\text{-}DR^-$) that contains the cells capable of initiating in vitro long-term BM cultures, the primitive HPP-CFC, BFU-MK, and the CFU-B1. $CD34^+$ $HLA\text{-}DR^-$ BM cells, which represent 0.08% to 0.45% of total nucleated BM cells,[10] constitute a fraction of adult BM cells enriched for primitive hematopoietic precursor cells.[5,6] This characteristic of $CD34^+$ $HLA\text{-}DR^-$ cells was demonstrated by their ability to initiate and sustain chimeric human hematopoiesis in three animals (4033, 4035, and 4038, Table 1). The fact that we detected in two animals (4033 and 4035) transplanted with viable $CD34^+$ $HLA\text{-}DR^-$ cells only at least an 18-fold increase in the number of human cells used as a graft and that hematopoietic colonies containing cells with a human karyotype were generated from their chimeric BM cells indicate that proliferation of and most likely engraftment with human $CD34^+$ $HLA\text{-}DR^-$ cells had occurred in both fetuses. Because the only viable cells that these animals received were $CD34^+$ $HLA\text{-}DR^-$, these studies suggest that BM cells bearing this phenotype are capable of sustaining hematologic reconstitution in vivo. It is important to emphasize that it is also possible that the in vitro colony growth observed in the chimeric BM of these two animals may have resulted from a transient expansion of committed progenitor cells contained within the $CD34^+$ $HLA\text{-}DR^-$ fraction of BM cells. However, the magnitude of increase in the number of human cells detected in these two animals had most likely resulted not nly from the proliferation of committed progenitor cells, but also from the expansion of a group of cells capable of self-renewal.

In addition to the two fetuses in which engraftment was documented 30 days posttransplantation, on of the three sheep born alive (4038) was also a chimera, bringing the total number of sheep that had engrafted to three of sev n

Table 3. Hematopoietic Colony Formation by Fractionated and Unfractionated Chimeric BM Cells Obtained From Sheep 4038 2 Months After Birth

Source of Cells	Added Cytokines*			No. of Colonies per 5 × 10^4 Plated Cells				
				Erythrocytic			Granulocytic/Monocytic	
				CFU-E	BFU-E		CFU-GM	
	Epo	IL-3	GM-CSF	Day 6	Day 9	Day 14	Day 9	Day 14
Control sheep	+	–	–	15 ± 3	17 ± 13	1 ± 1	ND‡	ND
	–	+	+	ND	ND	ND	2 ± 1	2 ± 0
Sheep 4038	+	–	–	23 ± 2	18 ± 1	10 ± 4	ND	ND
	–	+	+	ND	ND	ND	13 ± 1	12 ± 1
Sheep 4038								
CD45⁺	–	+	+	ND	ND	ND	20 ± 0	40 ± 5
CD45⁻	–	+	+	ND	ND	ND	3 ± 1	3 ± 1
Human donor	+	–	–	43 ± 9	27 ± 2	61 ± 1	ND	ND
	–	+	+	ND	ND	ND	111 ± 4	120 ± 1
Human/sheep	+	–	–	13 ± 7	1 ± 1	2 ± 1	ND	ND
BM mixture†	–	+	+	ND	ND	ND	5 ± 1	6 ± 1

*Recombinant human cytokines were added at the following concentrations: Epo, 1 U/mL; IL-3, 1 ng/mL; GM-CSF, 1 ng/mL.

†Normal human donor BM cells were mixed with BM cells from the control sheep at a ratio of 5:95 and then plated out in semi-solid medium as described.

‡In cultures not receiving IL-3 and GM-CSF, the number of background CFU-GM–derived colonies was not determined (ND). Conversely, in cultures initiated in the absence of Epo, background erythroid colonies were not determined.

transplanted animals. The fact that sheep 4038 received, in addition to the 2 × 10^4 CD34⁺ HLA-DR⁻ cells, 1 × 10^6 complementary cells raises the issue that the complementary cells could have played an essential role in establishing the initial human hematopoiesis observed in this animal. Complementary cells, which in theory constitute the Fr 12-14 BM cells minus CD34⁺ HLA-DR⁻ cells (and therefore include CD34⁺ HLA-DR⁺ cells), constitute an enriched source of committed hematopoietic progenitor cells[25] with limited self-renewal capacity. It is therefore conceivable to attribute the first wave of hematopoietic reconstitution after BM transplantation to the complementary cells and to even assume that some of these cells contributed to the maintenance of sustained human hematopoiesis in this animal. The reasons for the inability to establish chim rism in the other two lambs born alive are unknown but are lik ly due to the low number of viable cells used as the hematopoietic graft.

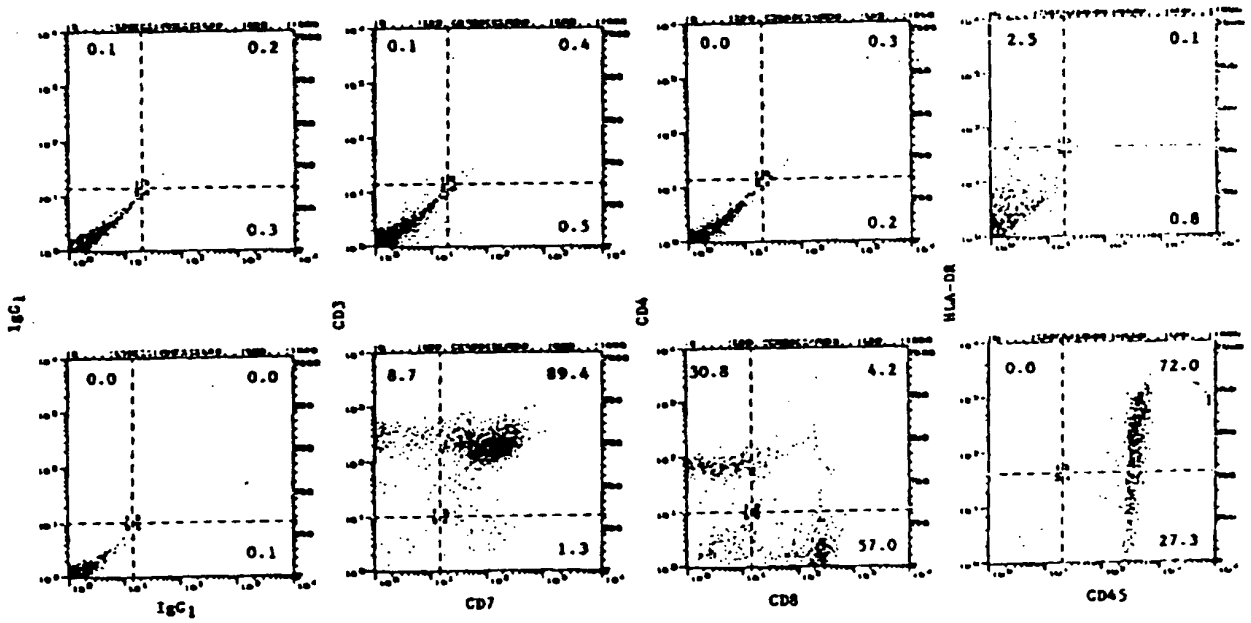

Fig 5. Phenotypic analysis of T-lymphocyte colonies generated from sorted CD45⁺ and CD45⁻ chimeric BM cells from sheep 4038 5 months posttransplantation. Cells from 15 to 20 colonies from each group were pooled, stained, and analyzed as described in the legend to Fig 1. The top row of four histograms represents the analysis of cells from colonies generated from CD45⁻ cells (sheep in origin), whereas the analysis of cells from colonies generated from CD45⁺ cells (human in origin) is shown in the bottom row of histograms. Only cells of human origin reacted with the MoAbs used in this analysis.

In the present study, a relatively small number of cells constituting the BM graft in these animals was sufficient to establish human hematopoiesis. Recent in utero BM transplants in the rhesus monkey[3] have used at least 9×10^6 and as many as 7×10^7 allogeneic fetal liver cells per fetus to achieve chimerism. Similar numbers of cells have been used in fetal sheep.[1,26] It is important, however, to point out that in these studies a higher degree of chimerism was attained in the surviving animals than what was observed in sheep 4038. The effective achievement of chimerism in our animals despite the low cell number in the graft is most likely due to the use of BM fractions enriched for hematopoietic progenitor cells at various stages of lineage commitment,[5-9] rather than unfractionated cell preparations. In the murine system, especially in studies using the SCID mouse, much higher numbers of human liver cells had to be used as a graft and only a transient wave of human T lymphocytes in the PB was detected.[27] In studies using 10^7 human fetal liver cells injected into SCID mice with a fetal human thymic implant, McCune et al[27] were only capable of detecting human T lymphocytes in the PB of these animals for up to 49 days posttransplantation. In these studies, only mice engrafted with human fetal liver cells, human fetal lymph node, and human fetal thymus were capable of producing human IgG. Kamel-Reid and Dick[28] infused *bg/nu/xid* mice with 5×10^6 to 5×10^7 adult human BM cells and were capable of detecting human macrophage progenitor cells in BM and spleen cells of the recipient mice. Although engraftment was detected in this system 5 weeks after the infusion of human BM cells, only slightly irradiated *bg/nu/xid* mice accepted the human graft and the level of chimerism was approximately 0.3% to 3% in the spleen and BM. Considering several factors that can negatively influence the degree of chimerism achieved in our model, such as the number of cells constituting the graft, the time at which chimerism was assessed after transplantation, and the fact that the graft was obtained from adult human BM, makes the degree of chimerism reported in this study consistent with what has been previously reported in other models.[27,28] In addition, the duration of persistence of chimerism in sheep 4038 is equal to or longer than what has been reported in other xenogeneic BM transplantation models.

The experiments described in this report indicate that in our model functional human hematopoiesis was evident for over 190 days posttransplantation. Similarly, human T lymphocytes displaying a normal array of cell surface markers could be recovered in this model after in vitro mitogen stimulation of chimeric BM cells. In view of the fact that it has been previously shown that the peripheral T-cell pool is normally maintained at homeostatic levels by postthymic expansion,[29-31] it becomes unclear from the present studies whether any of the recovered human T cells had underg ne a process of intrathymic education and maturation or not.

However, no human IgG was detected in sheep 4038. This observation may be due to the slow reconstitution of B cells and cooperative functions of T and B cells after BM transplantation in humans.[32] It is possible that the absence of human thymic or lymph node tissues in the chimeric animal lead to a breakdown in the antigen presenting cells/T-helper function/peripheral lymphoid organ network necessary for the generation of plasma cells and Ig production.

An exciting finding in our present studies is the absence of any clinically apparent GVHD in the chimeric animals after a period of observation ranging from 27 to 190 days after transplantation with xenogeneic grafts from an immunologically competent adult donor. Previously, HSC from preimmune fetuses have been shown to be capable of proliferating in a "non-self" environment without mounting a GVH reaction,[1,2] whereas grafts of allogeneic adult sheep BM cells induced fatal GVHD.[26,33] In the present report, fractions of human adult marrow are now shown to also be capable of serving as non-GVHD provoking grafts in preimmune fetuses in utero. It is important to emphasize that Fr 12-14 BM cells contain in general no more than 2% to 3% of detectable T-lymphoid elements as determined by flow cytometric analysis (Srour E.F., unpublished observations). It has been shown that T-cell–depleted adult allogeneic BM grafts failed to cause GVHD when transplanted into fetal sheep in utero.[33] However, it is difficult to reconcile the absence of GVHD in sheep 4038 and the possibility that the recovered human T cells from chimeric BM and PB had resulted from postthymic expansion of infused mature human T cells. Therefore, the absence of GVHD in sheep 4038 favors the hypothesis that it is possible that at least partial intrathymic education and maturation of human T-lymphocyte progenitor cells had taken place. It is also possible that the absence of GVHD might be due to the lack of development of human antigen-presenting cells that have been shown to be essential for the development of a human antimouse xenogeneic cytotoxic response[34] or to the fact that a xenogeneic immune response is, in general, weaker than an allogeneic response, as previously reported.[35]

The preliminary results gleaned from these experiments suggest that the model described in this report may prove useful in assessing the ability of various human BM subpopulations of sustaining long-term in vivo hematopoiesis. These studies also raise the possibility of using cell populations from adult human marrow and across HLA barriers for in utero marrow grafts to correct a variety of congenital metabolic and/or hematologic abnormalities. Successful corrections of congenital anomalies have already been accomplished in mice[36] and humans[37] using fetal liver HSC for in utero transplantation. The ability to use adult marrow cells for in utero transplantation will surely enhance the feasibility of this potentially useful treatment modality.

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